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(54) Title: LIPID CONSTRUCTS FOR CYTOPLASMIC DELIVERY OF AGENTS (57) Abstract A lipid construct comprising an aminomannose derivatized cholesterol suitable for targeting smooth muscle cells and tissue. Preferred formulations contain 6-(cholest-5-en-3 β -yloxy)hexyl-6-amino-6-deoxy-1-thio- α -D-mannopyranoside in liposome formulations wherein the formulations are delivered generally to arteries using percutaneous transluminal coronary angioplasty procedures. These formulations have applications in the reduction of restenosis.		

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LIPID CONSTRUCTS FOR CYTOPLASMIC
DELIVERY OF AGENTS

10

FIELD OF THE INVENTION

This invention relates generally to pharmaceutical sciences and more particularly to the production of lipid constructs for the delivery of drugs to the cytoplasm of a cell and in particular to vascular smooth muscle tissue. A preferred use of this invention relates to the administration of these
15 constructs for the prevention of restenosis following angioplasty.

BACKGROUND OF THE INVENTION

In order for many drugs and imaging agents to have therapeutic potential it is necessary for them to be delivered to the proper location in the body. In many cases, agents are ineffective because it is impossible, with the
20 available technology, to deliver them to the proper locations within a biological organism. Thus, a main focus in pharmaceutical research is in the delivery of agents to tissues and cells.

An area of development aggressively pursued by researchers is in the delivery of agents not only into a cell but into the cell's cytoplasm and further
25 yet, into the nucleus. This area of research is being pursued in particular for delivery of biological agents such as DNA, RNA, ribozymes and proteins. Not only do these materials hold great promise as therapeutic agents, but some workers believe that in some diseases they may act as the "magic bullet" i.e., curing an illness without deleterious side effects. Examples of therapeutic
30 pursuits include the application of antisense technology. Briefly, the strategy in antisense technology is to deliver an agent such as DNA oligonucleotide that binds to sites on messenger RNA (mRNA) which directs the production of proteins related to disease. Other strategies include the use of triplex agents which bind to the double helix to interfere with transcription,

5 preventing the production of mRNA. In spite of these developments, numerous "delivery" problems have surfaced.

One major problem encountered is that effective delivery of oligonucleotides across cell membranes is difficult, if not impossible. Another problem facing workers is that oligonucleotides are degraded by intracellular
10 and extracellular enzymes such as exonucleases and endonucleases. In order to overcome these problems, workers have modified the typical phosphodiester form of oligonucleotides. For example, modifications have included the creation of phosphorothioate and methylphosphonate oligonucleotides. Methylphosphonate oligonucleotides contain uncharged
15 sectors which increase intracellular uptake and resist enzymatic degradation. Phosphorothioate oligonucleotides are negatively charged but are resistant to endonucleases. Although these modified oligonucleotides show promise as therapeutics, there is no evidence of *in vivo* success. Further, workers have not provided a delivery vehicle capable of introducing a sufficient quantity of
20 oligonucleotides into the cell such that it has therapeutic effect. Since it is desirable to load a cell with numerous oligonucleotides, workers have begun to focus their efforts on developing a delivery vehicle which not only acts to deliver an agent into the cytoplasm and nucleus of a cell, but also delivers oligonucleotides in large numbers.

25 Cationic liposomes have been used to introduce DNA into vascular cells. Plautz, G.E., *et al.*, "Liposome Mediated Gene Transfer into Vascular Cells", *J. Liposome Research* 3(2):179-199 (1993); Felgner, P.L., *et al.* "Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure", *Proc. Natl. Acad. Sci.* 84:7413-7417 (1987). For example, Lipofectin, liposomes
30 formed from a mixture of N-[2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE), has been used to transduce recombinant genes into coronary arteries *in vivo*. Another

5 example of a cationic liposome used to introduce genes into DNA is a liposome preparation containing DC-chol, 3b[N-9N'N'-dimethylaminoethane)-carbamoyl] cholesterol and DOPE. Other efforts have been directed toward the use of modified retroviruses and cationic liposomes for gene transfer.

Liposomes in general have been known. Liposomes are microscopic
10 vesicles made from phospholipids, which form closed, fluid filled spheres when dispersed with aqueous solutions. Phospholipid molecules are polar, having a hydrophilic head and two hydrophobic tails consisting of long fatty acid chains. Thus, when a sufficient concentration of phospholipid molecules are present in aqueous solutions, the tails spontaneously associate to exclude
15 water while the hydrophilic phosphate heads interact with water. The result is a spherical, bilayer membrane in which the fatty acid tails converge in the interior of the newly formed membrane, and the polar heads point in opposite directions toward an aqueous medium. These bilayer membranes thus form closed spheres known as liposomes. The polar heads at the inner surface of
20 the membrane point toward the aqueous interior of the liposome and, at the opposite surface of the spherical membrane, the polar heads interact with the surrounding aqueous medium. As the liposomes are formed, water soluble molecules can be incorporated into the aqueous interior, and lipophilic molecules may be incorporated into the lipid bilayer. Liposomes may be either
25 multilamellar, like an onion with liquid separating many lipid bilayers, or unilamellar, with a single bilayer surrounding an aqueous center.

Methods for producing liposomes are well known in the art, and there are many types of liposome preparation techniques which may be employed to produce various types of liposomes. These can be selected depending on
30 the use, the chemical intended to be entrapped, and the type of lipids used to form the bilayer membrane. The requirements which must be considered in producing a liposome preparation are similar to those of other controlled

- 5 release mechanisms. They are: (1) a high percent of chemical entrapment; (2) increased chemical stability; (3) low drug toxicity; (4) rapid method of production; and (5) a reproducible size distribution.

The first method described to encapsulate drugs or other chemicals in liposomes involved the production of multilamellar vesicles (MLVs).

- 10 Liposomes can also be formed as unilamellar vesicles (UVs), which generally have a size less than 0.5 μm (μm , also referred to as "microns"). There are several techniques known in the art which are used to produce unilamellar liposomes.

- Smaller unilamellar vesicles can be formed using a variety of techniques, such as applying a force sufficient to reduce the size of the liposomes and or produce smaller unilamellar vesicles. Such force can be produced by a variety of methods, including homogenization, sonication or extrusion (through filters) of MLVs. These methods results in dispersions of UVs having diameters of up to 0.2 μm , which appear as clear or translucent suspensions. Other standard methods for the formation of liposomes are know in the art, for example, methods for the commercial production of liposomes include the homogenization procedure described in U.S. Patent No. 4,753,788 to Gamble, a preferred technique, and the method described in U.S. Patent 4,935,171 to Bracken, which are incorporated herein by reference.

- 25 Another method of making unilamellar vesicles is to dissolve phospholipids in ethanol and inject them into a buffer, whereby the lipids will spontaneously rearrange into unilamellar vesicles. This provides a simple method to produce UVs which have internal volumes similar to that of those produced by sonication (0.2-0.5 L/mol of lipid). Another common method for producing small UVs is the detergent removal technique. Phospholipids are solubilized in either ionic or non-ionic detergents such as cholates, Triton X-100, or n-alkylglucosides. The drug is then mixed with the solubilized
- 30

5 lipid-detergent micelles. Detergent is then removed by one of several techniques: dialysis, gel filtration, affinity chromatography, centrifugation or ultrafiltration. The size distribution and entrapment efficiencies of the UVs produced this way will vary depending on the details of the technique used.

The therapeutic uses of liposomes include the delivery of drugs which
10 are normally toxic in the free form. In the liposomal form the toxic drug may be directed away from the sensitive tissue and targeted to selected areas.

Liposomes can also be used therapeutically to release drugs, over a prolonged period of time, reducing the frequency of administration. In addition, liposomes can provide a method for forming an aqueous dispersion
15 of hydrophobic drugs for intravenous delivery.

When liposomes are used to target encapsulated drugs to selected host tissues, and away from sensitive tissues, several techniques can be employed. These procedures involve manipulating the size of the liposomes, their net surface charge as well as the route of administration. More specific
20 manipulations have included labeling the liposomes with receptors or antibodies for particular sites in the body. The route of delivery of liposomes can also affect their distribution in the body. Passive delivery of liposomes involves the use of various routes of administration, *e.g.*, intravenous, subcutaneous and topical. Each route produces differences in localization of
25 the liposomes. Two methods used to actively direct the liposomes to selected target areas are binding either antibodies or specific receptor ligands to the surface of the liposomes. Antibodies are known to have a high specificity for their corresponding antigen and have been shown to be capable of being bound to the surface of liposomes, thus increasing the target specificity of
30 the liposome encapsulated drug.

Since the chemical composition of many drugs precludes their intravenous administration, liposomes can be very useful in adapting these

5 drugs for intravenous delivery. Furthermore, since liposomes are essentially hollow spheres made up of amphipathic molecules, they can entrap hydrophilic drugs in their aqueous interior space and hydrophobic molecules in their lipid bilayer. Unwanted molecules that remain in the dispersion external to the liposomes, such as untrapped agents, are removed by
10 column chromatography or ultrafiltration. Although methods for making liposomes are well known in the art, it is not always possible to determine a working formulation without experimentation.

United States Patent No. 4,310,505, incorporated by reference, discloses lipid vesicle formulations containing amino sugar derivatives of
15 cholesterol as cell-surface receptor analogs. The vesicles were found to release their contents in a controlled manner, and in some cases, to be rapidly concentrated in the lymphatic system and/or liver, lungs or spleen of the host. These liposomes were noted to be used in the treatment of lysosomal storage disease. Liposomes containing amino-sugar derivatives and have been
20 shown to localize in aggregates of polymorphonuclear leukocytes. Mauk, M.R. *et al.*, "Vesicle Targeting: Timed Release and Specificity for Leukocytes in Mice by Subcutaneous Injection" *Science* 207:309-311 (1980).

The delivery problems noted above become manifest in treating a disease such as arteriosclerosis, a disease associated with the hardening or
25 narrowing of arterial walls and the leading cause of death in Western Society. The majority of these deaths is caused by atherosclerosis, a type of arteriosclerosis characterized by lipid deposits in the intima of large and medium size arteries. These deposits or lesions are among three classes: fatty streaks, fibrous plaques and complicated lesions. Fatty streaks, probably the
30 earliest lesions of atherosclerosis, are generally characterized by a build-up of lipid-filled smooth muscle cells, macrophage and fibrous tissue in the intima. Advanced atherosclerosis gives rise to fibrous plaques, which are raised

5 lesions and elevated areas of intimal thickening. These lesions consist of a central core of extracellular lipid and necrotic cell debris (gruel) covered by a fibromuscular layer containing large numbers of smooth muscle cells, macrophage, and collagen. The third class, known as the complicated lesion, is characterized by calcified fibrous plaques. Stenosis, or narrowing, of
10 vascular passages can result from gradual occlusion as the plaques thicken and thrombi form.

Atherosclerosis and stenotic vascular lesions are typically treated through the use of percutaneous transluminal coronary angioplasty (PTCA) procedures. These methods, which include balloon angioplasty, the
15 atherectomy catheter, the excimer laser, and the rotablator, are used to dilate the stenosed blood vessels. However, the efficacy of PTCA is limited by the development of restenosis, or renarrowing, of the treated area following PTCA due to neointima formation. Approximately 25% of successful first-time angioplasty procedures must be repeated within 6 months and an
20 additional 10% of the patients must later undergo coronary bypass surgery because of restenosis. A discussion of the causative elements of restenosis appears in Bone, R.C., ed., "Restenosis after Coronary Angioplasty," *Disease-A-Month*, 39:616-670 (1993).

PTCA procedures often cause collateral injury to the arterial wall,
25 which triggers a "healing" process involving a series of physiologic events that ultimately results in neointima formation. Neointima formation is a multi-step process involving a complex interaction among several growth factors which promote vascular smooth muscle cell (VSMC) proliferation and migration. In this process, erosion of the intima, the single continuous layer
30 of endothelial cells lining the arterial walls, during angioplasty promotes platelet aggregation. The platelets adhere to the arterial wall and release growth factors (e.g., platelet-derived growth factor, thrombin, basic fibroblast

5 growth factor and transforming growth factor) which initiate the migration of VSMC to the intima and promote the proliferation of VSMC comprising the tunica media. Growth factor-induced VSMC proliferation involves, in turn, a sequential activation of intracellular proteins which promote cell-cycle progression. The next step of the process is associated with an inflammatory
10 response, evidenced by an invasion of inflammatory cells such as monocytes, macrophage, and other white blood cells. These inflammatory cells further induce and stimulate growth of VSMC in the media, which subsequently migrate into the intimal space and produce large amounts of extracellular proteolytic matrix material, forming a neointima layer which represents the
15 lesion. It appears that endothelium then grows over the injured area and restenosis results.

Considerable research has been undertaken to identify agents which prevent or reverse restenosis. For example, many agents have been examined in the prevention of restenosis. A comprehensive review of therapeutics
20 potentially useful in the treatment of restenosis appears in Herrman, Jean-Paul R. *et al.* "Pharmacological Approaches to the Prevention of Restenosis Following Angioplasty, The Search for The Holy Grail?" (parts I and II) *Drugs* 46 (1):18-52, 46 (2):249-262, 1993.

Other drugs considered for inhibiting restenosis include, but are not
25 limited to, oligonucleotides (e.g., antisense gene therapy), protein kinase C, endothelial growth factor and anti-platelet activating agents. Efforts to prevent restenosis utilizing systemic administration of such drugs, however, have been hampered by generalized toxicity at effective dosages.

Furthermore, successful therapy for preventing restenosis has been thwarted
30 by the complexity of the physiological processes responsible for neointima formation as well as the inability to deliver effective quantities of drugs to the site of injury, due to inefficient cellular uptake and lack of specificity (i.e.,

5 targeting) to VSMC. One possible mechanism for the failure of drugs to provide effective prevention of restenosis is that the agents never reach the tissue targeted. For example, the candidate agents may simply be washed away from the treatment areas by blood flow.

Gene therapy, using antisense oligonucleotides which effectively
10 "shut off" the genes involved in neointima formation, is a promising approach to the problem of restenosis. However, due to the multiplicity of growth factors involved in neointima formation, selective inhibition of any single growth factor is unlikely to completely prevent lesion formation. Accordingly, works have shown inhibition of neointima hyperplasia in a rat
15 carotid model following angioplasty injury by administration of two antisense oligonucleotides (AS-oligos) which block the cell-cycle regulatory genes for proliferating-cell nuclear antigen (PCNA) and p34^{cdc2} (cdc2). Morishita, R. *et al.* "Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal
20 hyperplasia" *Proc. Natl. Acad. Sci.* 90:84774 (1993). PCNA, a nuclear protein required for DNA synthesis by DNA polymerase Δ , and cdc2, a serine/threonine protein kinase, are primary components of the cell-cycle progression which regulate VSMC proliferation. The AS-oligos were constructed to be directed to the translation initiation sites of PCNA and
25 cdc2. Cellular uptake was enhanced and transfection stability of the AS-oligos increased by complexing the phosphorothioate AS-oligos with liposomes and the protein coat of inactivated Sendai hemagglutinating virus of Japan (HVJ). Morishita *et al.* observed more rapid uptake and a 10-fold increase in transfection efficiency of AS-oligos or plasmid DNA than
30 standard lipofection or passive uptake methods. However, specific targeting of the HVJ AS-oligos liposome complex to VSMC was not reported. It is generally desired then to develop targeting specificity of drug carriers to

5 VSMC since that would result in greater uptake by the smooth muscle cells at the site of injury and would permit the administration of higher, more effective dosages. Furthermore, it is advantageous to avoid the use of viral proteins in drug carriers as they present a potential biological hazard to humans.

Thus, it is an object of the present invention to provide a lipid
10 construct that is superior and more effective in delivering agents into the cytoplasm and nucleus of a cell. It is yet another object of the present invention to provide for the targeting of an agent to vascular smooth muscle tissue. It is still yet a further object of this invention to provide for a less toxic and more efficacious treatment of such diseases as restenosis, viral diseases
15 and cancer.

SUMMARY OF THE INVENTION

The invention provides for the delivery of an imaging or therapeutic agent into the cytoplasm of a cell by delivering the lipid construct to a mammal wherein the lipid construct comprises an aminomannose derivatized
20 cholesterol. The delivery of the agents could be done *in vitro* as well as *in vivo*. This includes delivery of agents to cells, tissues or organs *in vitro* and reintroduction of the cells, tissues or organs *in vivo*. It also includes the transfection of cultured cells *in vitro* for the purpose of conferring desirable properties in these cells, including enhanced, decreased or novel production
25 of proteins.

Lipid constructs, for purposes of this invention, are complexes containing lipids, phospholipids, or derivatives thereof comprising a variety of different structures which lipids are known to adopt in aqueous suspension. These structures include, but are not limited to, micelles,
30 liposomes, lipid ribbons or sheets, and may be complexed with a variety of drugs and adjuvants which are known to be pharmaceutically acceptable. Common adjuvants include cholesterol and α -tocopherol, among others. The

5 lipids may be used alone or in any combination which one skilled in the art would appreciate to provide the characteristics desired for a particular application. In addition, the technical aspects of lipid complex and liposome formation are well known in the art and any of the methods commonly practiced in the field may be used for the present invention.

10 The present invention further comprises a method for targeting vascular smooth muscle tissue and cells comprising the delivery of an agent to the cell using a lipid construct comprising an aminomannose derivatized cholesterol. This approach is advantageous over the prior art since it provides targeting but does not involve the use of viral products. The
15 aminomannose-containing lipid constructs exhibit dramatically enhanced *in vitro* and *in vivo* targeting to VSMC, compared to identical constructs not containing aminomannose. While not wishing to be bound by any particular theory, it appears that the increased targeting results from specific mannose-receptor-mediated endocytosis or fusion of the lipid constructs.

20 In another aspect of the invention, the lipid constructs contain drugs which inhibit neointima formation and are administered to prevent restenosis following PTCA procedures. Since restenosis is caused generally from the accumulation of Vascular Smooth Muscle Cells, the targeting of such cells is critical during PTCA procedures.

25 Also provided herein is a method of delivering a lipid composition into a vascular smooth muscle cell by delivering a lipid construct to a mammal comprising aminomannose derivatized cholesterol.

The preferred composition for carrying out the invention includes a lipid construct comprising an aminomannose derivatized cholesterol. Further
30 embodiments of the invention include the construct having a cationic lipid wherein the total cationic lipid is greater than or equal to 5% of total lipid and the lipid construct further comprises a therapeutic or imaging agent. The

- 5 aminomannose derivatized cholesterol is included in the definition of a cationic lipid.

The invention is useful for inhibiting restenosis in a mammal by delivering a lipid construct to the mammal wherein the lipid construct comprises an aminomannose derivatized cholesterol and a cationic lipid
10 wherein the total cationic lipid is greater than or equal to 5% of total lipid wherein the lipid construct further comprises an agent in an amount effective for said inhibition. The 5% cationic lipid could include only aminomannose derivatized cholesterol.

Another aspect of the invention comprises the cytoplasmic delivery of
15 an agent into the cytoplasm of a cancer cell.

Brief Description of the Drawings

Figure 1 shows a freeze fracture micrograph of a lipid construct-containing oligonucleotides.

Figure 2 shows a confocal micrograph of fluorescently labeled chimeric
20 oligonucleotide taken at 2.5 hours.

Figures 3A & 3B show a confocal micrograph of DOPE:Amch complexed with labeled chimeric oligonucleotide taken at 2.5 hours and 16 hours respectively.

Figures 4A & 4B show a confocal micrograph of DSPC:Chol
25 complexed with labeled chimeric oligonucleotide taken at 2.5 hours and 16 hours respectively.

Detailed Description of the Invention

The present invention relates to the formation of a lipid construct comprising an aminomannose derivatized cholesterol. A preferred
30 representative of this compound is 6-(cholest-5-en-3 β -yloxy)hexyl-6-amino-6-deoxy-1-thio- α -D-mannopyranoside (Amch).

Of course, any number of lipids may be used in combination with

5 aminomannose derivatized cholesterol. Examples of lipids which may be used successfully include, but are not limited to: dimyristoyl triethylamine propane (DMTAP), dimyristoyl dimethylammonium propane (DMDAP), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-dioleoyl-3-dimethylammonium propane (DODAP) and dioleoyl-sn-glycero-3-ethyl phosphatidylcholine
10 (DGE), hydrogenated soy and egg phosphatidyl choline (HSPC, HEPC) distearoyl phosphatidylcholine (DSPC), Dimyristoyl phosphatidylcholine (DMPC), dilauryl phosphatidylcholine (DLPC), dioleoyl phosphatidylcholine (DOPC), phosphatidyl ethanolamine (PE), dimyristoyl phosphatidylethanolamine (DMPE), dioleoylphosphatidylethanolamine
15 (DOPE), cholesterol, N-[2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 3b[N-(N'N'-dimethylaminoethane)-carbonyl] cholesterol (DC-Chol). These lipid constructs may also be used to treat or prevent cancer or viral infections by facilitating delivery of oligonucleotide therapeutic agents to the cellular cytoplasm.

20 These lipids may be used in any suitable combination with an aminomannose derivatized cholesterol. Preferred lipid formulations are listed in Tables 1 & 2. The following formulations give rise to optimal results in preventing restenosis: DOPE:Amch (1:1 molar ratio) having a lipid to oligonucleotide ratio (w/w) less than 5:1 and preferably between 1.25:1 &
25 0.5:1. The preferred size of these construct is range from 10nm to 250nm preferably at 50nm. Preferred lipids used in the constructs comprise cationic lipids, preferably greater than or equal to 5% total. The preferred lipid construct is a unilamellar liposome as shown in Figure 1 wherein the liposome is shown prepared having a formula DOPE:Amch (1:1 molar ratio).

30 The lipid constructs preferred for use in delivering oligonucleotides which inhibit tumor growth comprise HSPC:Chol:Amch in a mole ratio of 16:8:1.

5 The preferred lipid constructs are prepared by first hydrating a mixture of lipids in suitable buffer such as 9% sucrose. Prior to hydration, it is preferable that a lipid powder or film of the lipid mixture is formed, for example, by spray-drying or rotary evaporative techniques.

 One preferred use of the invention is in the treatment of restenosis, and
10 further for the prevention of neointima formation. Agents that may be used include the following: antithrombotic agents, antiplatelet agents, antiproliferative agents, anti-inflammatory agents, calcium antagonists, lipid lowering drugs, and antibodies to growth factors, inositol diphosphate, and antisense compounds. Some of these agents include coumadin, heparin, low
15 molecular weight heparins, hirudin, aspirin, dipyridamole, thromboxane A₂, thromboxane A₂ synthetase inhibitor, thromboxane A₂ receptor blockers, ridogrel, sulfinpyrazone, dextran, prostacyclin or prostacyclin analogues, prostaglandin E₁, ticlopidine, serotonin antagonists, glycoprotein IIb/IIIa receptor blockers, angiotensin converting enzyme inhibitors, colchicine,
20 platelet-derived growth factor antagonists, angiopeptin, somatomedin, octreotide, cytostatic, corticosteroids, ibuprofen, nifedipine, nicardipine, diltiazem, verapamil, fish oils, (such as omega 3 fatty acids, eicosapentaenoic acids, and docosahexaenoic acids), lovastatin, postangioplasty, colestipol, and antibodies. Microparticles have been noted as a potential microcarrier
25 drug delivery system in order to prevent the rapid elimination by the increase network of vasa vasorum and atherosclerotic lesions that cause early outward diffusion. Red blood cells have also been investigated as a microcarrier system.

 The preferred agents used are oligonucleotides that inhibit neointima
30 formation and prevent restenosis. The preferred size of the oligonucleotides is from 15 mer to 30 mer, although sizes up to 80 mer may be associated with the lipid construct taught in this invention.

5 The present invention is also useful for blocking cell-cycle regulatory genes such as proliferating-cell nuclear antigen (PCNA), p34cdc2 (cdc2), and cdk2 (cyclin-dependent kinases). Antisense oligonucleotides directed toward translation sites of these genes provide optimum results. In the treatment of cancer, such antisense oligonucleotides corresponding to the bcl-2 or c-myc
10 mRNA provide significant benefits.

 This invention has particular advantages for complexing aminomannose derivatized cholesterol with oligonucleotide ligands generated by the SELEX[®] technology as described in U.S. Patent Number 5,270,163 and incorporated herein by reference.

15 This invention will be more fully understood by references to the following examples, which are intended to be illustrative of the invention, but not limiting thereof.

Example 1

Liposomal Targeting and Uptake of Smooth Muscle Cells

20 A. Preparation of Liposomes.

Two liposome formulations were prepared. A lipid film containing distearoyl phosphatidylcholine (DSPC) and cholesterol (Chol) in a 2:1 molar ratio was prepared. A second lipid film containing DSPC, Chol and aminomannose derivatized cholesterol (Amch) in a molar ratio of about 2:0.96:0.04 was also
25 prepared. The lipid films were formed by dissolving the lipids in chloroform, mixing and then evaporating off the chloroform. The lipid films were radiolabeled with ³H-cholesteryl hexadecyl ether and/or fluorescently labeled with the lipophilic fluorescent probe, octadecyl rhodamine. The ³H-cholesterylhexadecyl ether (5 μ Ci) was added during the formation of the lipid
30 film. The lipids were hydrated in 100 mM NaCl and 25 mM Phosphate buffer pH 7.4 and probe sonicated for approximately 10 minutes to form unilamellar vesicles with an average diameter of 70 nm \pm 20 nm. The octadecyl rhodamine

5 was incubated with the liposomes for 20 minutes to fluorescently label the liposomes.

B. *In Vitro* Measurement of Uptake by Smooth Muscle Cells.

The ³H-cholesterylhexadecyl ether labeled liposomes were incubated with confluent rat aortic vascular smooth muscle cells for 5, 30, and 60 minutes.

10 The uptake of the liposomes was measured by scintillation counting and reported in Table 1 as counts per minute (cpm) per microgram of cell protein. All time points were done in triplicate. Fluorescence microscopy and autoradiography were performed to qualitatively detect the association of the liposomes with the vascular smooth muscle cells.

15

TABLE 1

Uptake of Labeled Liposomes by Vascular Smooth Muscle Cells
In CPM/ μ G Protein

	0'	5'	30'	60'
DSPC:Chol	1.35	3.63	4.22	6.51
DSPC:Chol:Amch	1.22	25.17	40.35	52.53

C. *In Vivo* measurement of Uptake by Smooth Muscle Cells.

20 White New Zealand rabbits were anesthetized and both femoral arteries were exposed. A balloon catheter was advanced into the iliac artery through a cut-down of the femoral artery. The catheter consisted of a metallic plate and tubular extrusions (delivery nipples) occupying one third the circumference of a balloon. The delivery nipples of the balloon deepened about 0.1 mm into
25 the arterial wall. The balloon of the catheter was inflated to 1.5 atm. One ml of aminomannose liposomes was delivered into the catheter against the vessel wall with 100 psi within three seconds. Both iliac arteries were used in all animals. The rabbits (6 arteries/group) were sacrificed at 0.5, 1, 4, 24 and 168 hours. Rabbits injected with normal saline or octadecyl rhodamine served as

- 5 controls (4 arteries/group). The iliac arteries were snap frozen in liquid nitrogen and the rhodamine fluorescence was measured using an Olympus fluorescence microscope.

At thirty minutes the liposomes were accumulated under the adventitia. At four hours, the liposomes began to distribute in the medial layer. At
10 twenty-four hours, the liposomes were distributed in a heavy homogeneous pattern throughout the width of the arterial wall. At one week, the intima remained stained and the liposomes were redistributed into the adventia with the media being clear. The blood vessels of rabbits are somewhat peculiar in that the intima is extremely thin. Therefore, any intima seen after
15 catheterization is assumed to be neointima, formed as a result of migration of VSMC from the media and consequent release of extracellular matrix. By 24 hours, the liposomes redistribute throughout the vessel. At one week the fluorescent dye is clearly seen within the neointima and not the media. Liposomes associated with VSMC within the media migrated across the
20 internal elastica into the neointima.

The above examples establish that lipid constructs comprising an aminomannose derivatized cholesterol target vascular smooth muscle cells (VSMC). Continuing fluorescence microscopy confirmed delivery of the lipid construct to VSMC, evidenced by binding of aminomannose derivatized
25 liposomes to the VSMC tissue. Autoradiography confirmed delivery into VSMC, evidenced by intracellular localization of the lipid within the VSMC.

Example 2

Liposomes containing greater than 5% cationic lipid were prepared. The lipids were hydrated in sterile-filtered 9% sucrose, heated for 0.5 minutes
30 at 65°C and sonicated. Sizing was done by microtrac analysis. Oligonucleotides (2328) GTCCTCCATAGTTACTCA (SEQ ID NO.: 1), (2342) GATCAGGCGTGCCTCAAA (SEQ ID NO.: 2),

- 5 (2616) ACTCATTGATACCTCCTG (SEQ ID NO.: 3) and
 (2617) AAACCTCCGTGCGGACTAG (SEQ ID NO.: 4) were added to the
 liposomes and heated at 65°C for 10 minutes. Table 2 lists all cationic
 formulations, lipid concentrations and liposomes sizes.

TABLE 2

CATIONIC FORMULATIONS

10

Formulation#*	Lipid Conc.(mg/mL)	Size(μm)
DOTAP:DOPE:Amch (1:1:0.5)	1	0.1296 ± 0.1446
DODAP:DOPE:Amch (1:1:0.5)	1	1.2961 ± 1.0803 (34%) 0.0450 ± 0.0434 (66%)
DOPE:Amch (1:1)	1	0.8619 ± 0.6654 (17%) 0.0942 ± 0.1699 (83%)
DMPE:Amch (1:1)	1	0.2734 ± 0.1955 (44%) 0.0719 ± 0.0604 (58%)
DGE:DOPE:Amch (1:1:0.5)	1	2.10 ± 0.482 (35%) 0.0435 ± 0.044 (65%)
DGE:DOPE:Amch (1:1:0.5)	1	1.4785 ± 0.5654 (60%) 0.1566 ± 0.1430 (20%) 0.0403 ± 0.0230 (20%)
DOPE:Amch (1:1)	1	0.8325 ± 0.7217 (39%) 0.0594 ± 0.1035 (61%)
DMPE:Amch (1:1)	1	1.5735 ± 0.6965 (18%) 0.0547 ± 0.0917 (82%)
DMPE:Amch (1:1.5)	1	1.0709 ± 0.3329 (20%) 0.0445 ± 0.0445 (74%) 0.0092 ± 0.0019 (6%)
DOPE:Amch (1:1)	2.85	0.0375 ± 0.0510
DMTAP:DMPE:Amch (1:1:0.5)	3.1	0.0158 ± 0.0261
DMDAP:DMPE:Amch (1:1:0.5)	3.1	1.6531 ± 1.1338 (5%), 0.3152 ± 0.2193 (18%) 0.0551 ± 0.0542 (77%)
DMTAP:DMPE:Amch (1:1:1)	3.1	1.7614 ± 0.5416 (1%) 0.0123 ± 0.0090 (99%)

5

Formulation#*	Lipid Conc.(mg/mL)	Size(μ m)
DOPE:Amch (1:1)	2.85	0.0431 \pm 0.0437 (60%) 0.0218 \pm 0.0053 (19%) 0.0116 \pm 0.0020 (21%)
DOPE:Amch (1:1)	3.1	1.1795 \pm 0.5194 (2%) 0.0416 \pm 0.0761 (98%)
DOTAP:DOPE:Amch (1:1:1)	3.72	0.2333 \pm 0.1677
DODAP:DOPE:amino (1:1:1)	3.72	0.888 \pm 0.5061 (11%) 0.1172 \pm 0.0988 (59%) 0.0437 \pm 0.0179 (30%)
DOPE:Amch (1:1)	3.1	0.0438 \pm 0.0719
DcChol:DOPC:Amch (0.25:1:0.25)	4.8	0.3135 \pm 0.1055 (2%) 0.0446 \pm 0.0603 (96%)
DcChol:DOPC (0.5:1)	2.5	1.1016 \pm 1.6545 (30%) 0.0437 \pm 0.0469 (70%)
DcChol:DOPE:Amch (0.5:1:0.5)	3.1	0.0327 \pm 0.0534
DcChol:DOPE (1:1)	2.7	1.5865 \pm 0.4214 (24%) 0.0414 \pm 0.0301 (27%) 0.0095 \pm 0.0018 (49%)
DOTAP:DOPE:Amino (1:1:0.5)	3.1	0.0496 \pm 0.0508
DOPE:Amino (1:1)	4.65	0.0428 \pm 0.0718
DOPE:Amino (1:1.5)	3.0	1.4996 \pm 0.4880 (12%) 0.0522 \pm 0.0840 (88%)
DOTAP:DOPE:Amch (1:1:1.5)	3.0	1.6306 \pm 0.8100 (13%) 0.0343 \pm 0.0512 (87%)
DOTAP:DOPE:Amch (1.3:1:1.2)	3.0	1.9534 \pm 0.7815 (14%) 0.0541 \pm 0.0867 (86%)
DOPE:Amch (1:1.2)	3.1	0.3203 \pm 0.2097 (7%) 0.0396 \pm 0.0407 (93%)
DOTAP:DOPE:Amch (1:1:1.5)	3.1	0.0417 \pm 0.0363
DOPE:Amch (1:1)	3.1	0.0622 \pm 0.0783
DOSPA:DOPE:Amch (3:1:0.5)	0.75	1.0169 \pm 0.2413
DOTMA:DOPE:Amch (1:1:0.5)	0.8	0.0953 \pm 0.1004

Typically, liposomes were sonicated until greater than 85% of the liposomes were less than 300nm diameter. This allowed sterile filtration using

- 5 a 450nm cellulose acetate filter without significant liposome loss on the filter. All liposome formulations except DOTAP:DOPE:Amch (1:1:1), DODAP:DOPE:Amch (1:1:1) and DOSPA:DOPE:Amch were 10-100nm average diameter. DOTAP:DOPE:Amch (1:1:1) and DODAP:DOPE:Amch(1:1:1) averaged 100-200nm diameter; DOSPA:DOPE:Amch were greater than 1 μ m
10 and could not be sterile filtered.

Example 3

- Lipid constructs were prepared as in Example 2. Electron microscopy were obtained of liposomes prepared with DOPE:Amch (1:1, mole ratio) and mixed with oligonucleotides in a 5:1 lipid:oligo ratio and immediately frozen
15 (Figure 1). Figure 1 clearly indicates that unilamellar liposomes are formed (magnified 60,000x (1cm=167nm) with an average size of 62 ± 5 nm. Two 21-mer phosphorothioate oligonucleotides targeted to the CAT gene were complexed (and combined), with the lipid construct. The sequences of the oligonucleotides were (2520) TAGCTTCCTTAGCTCCTGCAT (SEQ ID NO.: 5) and (2519) TAGCTT CCGCAACTCTTGCAT (SEQ ID NO.: 6), both antisense
20 to a mRNA which codes for chloramphenicol acetyl transferase (CAT). Incorporation of the oligos into the cytoplasm should inhibit CAT activity for the proper plasmid only if it is a specific antisense effect. The extent of inhibition of restenosis was determined using either of two methods: a chloramphenicol acetyl transferase (CAT) assay or a PCNA/cdc2 assay.
25 Chloramphenicol is an antibiotic which blocks protein synthesis by inhibiting the peptidyl transferase activity of the 50S ribosomal subunit in prokaryotes; peptidyl transferase catalyzes the formation of peptide bonds. CAT is a bacterial drug-resistance gene which inactivates chloramphenicol by
30 acetylating the drug at one or both of its two hydroxyl groups.

In the CAT assay, two expression plasmids containing modified bacterial CAT genes were constructed. These plasmids were pG1000 and

5 pG1007 as taught in Reynolds, M.A. "Triple-Strand-Forming methylphosphonate oligonucleotides targeted to mRNA efficiently block protein synthesis," *Proc Acad. Sci.* 91:12433-12437 at 12434 (1994), with the following modifications; the amplified DNA fragment containing the sequence target site for oligonucleotides 2519 or 2520 was substituted for the

10 polypyrimidine insert. The two plasmids differed from one another by four bases in the translation initiation region targeted by the oligos. This gene is usually not found in eukaryotes; thus there is little or no background CAT activity in eukaryotic cells. Cell extracts were incubated with ^3H or ^{14}C chloramphenicol and n-butyryl coenzyme A. ^3H or ^{14}C chloramphenicol + n-

15 butyryl coenzyme A \rightarrow CAT \rightarrow ^3H or ^{14}C chloramphenicol-n-butyryl. The modified chloramphenicol partitioned into the xylene fraction while the unmodified chloramphenicol remained in the aqueous phase.

Cos 7 cells or A10 smooth muscle cells were plated the day before the experiment so that confluency was approximately 70% on the day of the

20 experiment. Formulations of lipofectin complexed with plasmid and lipid constructs complexed with phosphorothioate oligonucleotides were diluted in OptiMEM, a serum-free medium. The cells are washed once with OptiMEM and then incubated for 3 hr with 0.5 mL of the diluted liposome-oligo complexes. The liposomes were removed and washed with OptiMEM and

25 transfected for 3 hours with the target plasmid complexed with Lipofectin.

The plasmid complexes were removed and the cells were cultured overnight in complete medium. The cells were harvested approximately 16 hours after the addition of the target plasmid. Cell lysates were analyzed for CAT activity to assess inhibition and protein content. The results are

30 displayed in Table 3.

TABLE 3

Transiently Transfected COS 7 Cells

Formulation	Lipid:oligo	%Inhib. 1 μ M	0.5 μ M	0.1 μ M oligo
DOTAP:DOPE:Amch (1:1:0.5)	5:1	94, 91	94	64
DODAP:DOPE:Amch (1:1:0.5)	5:1	92, 90	93	64
DOPE:Amch (1:1)	10:1	90	85	39
DMPE:Amch (1:1)	10:1	89	85	32
DMPE:Amch (1:1)	5:1	88, 95, 95	79	25
DGE:DOPE:Amch (1:1:0.5)	5:1	84, 90	50	11
DMPE:Amch (1:1.5)	10:1	88	72	29
DMTAP:DMPE:Amch (1:1:0.5)	5:1	92	83	24
DMDAP:DMPE:Amch (1:1:0.5)	5:1	84	50	15
DMTAP:DMPE:Amch (1:1:1)	5:1	87	70	41
Lipofectin	2.5:1	93, 86	90	62

A stably inducible model was also evaluated using COS 7 cells and A10 smooth muscle cells. These cells were stably transfected with a plasmid containing an inducible CAT gene using LipofectAmine (DOSPA:DOPE 3:1 mole ratio). COS 7 and A10 smooth muscle cells were plated the day before the experiment, treated with the liposomes and washed as described above. The liposome-oligo complexes were removed, washed in OptiMEM and cultured overnight in medium containing CdCl₂, which induces protein production. The cells were harvested approximately 16 hours later and analyzed for CAT activity. Cells were treated with μ M oligo. The results are displayed in Tables 4 and 5.

The plasmid containing an inducible CAT gene is pG1041 which has a modified chloramphenicol acetyl transferase (CAT) gene inserted into pMEP4 (Clonotech). pG1041 was produced as follows:

A modified chloramphenicol acetyl transferase (CAT) gene producing wild-type CAT protein was amplified in a PCR reaction using the following primer:

5 5' primer:

Hind III

5' cac aag ctt gtc gac tcg agt gca gga gct aag gaa gct acc atg gag aag aag atc
act gga tat acc acc 3' (SEQ ID NO.: 7)

3' primer:

10 Not I

5' tta tgc ggc cgc tta cgc ccc gcc ctg cca 3' (SEQ ID NO.: 8)

The amplified fragment was cut using Hind III and Not I restriction enzymes
and ligated into Hind III/Not I cut pMEP4 (Clontech vector. The amino
terminal protein and 5' nucleotide sequences of the CAT gene in pG1041

15 compared to wild-type CAT are:

Wild-type CAT:

5' +1 3'
Met Glu Lys Lys Ile Ser Gly Tyr Thr Thr
(SEQ ID NO.: 9)

20 uuu uca gga gcu aag gaa gcu aaa aug gag aaa aaa auc acu gga uau acc acc
pG1041, UCAT: (SEQ ID NO.: 10)

5' +1 3'
Met Glu Lys Lys Ile Ser Gly Tyr Thr Thr
(SEQ ID NO.: 11)

25 agu gca gga gcu aag gaa gcu acc aug gag aag aag auc acu gga uau acc acc
(SEQ ID NO.: 12)

The first adenosine of the open reading frame is designated +1. The coding
region nucleotide base changes between wild-type and pG1041 are
conservative and do not change the amino acid sequence. Amino acid code:

30 Asp = aspartic acid, Arg = arginine, Glu = glutamic acid, Gly = glycine, Ile =
isoleucine, Lys = lysine, Met = methionine, Ser = serine, Thr = threonine, Tyr
= tyrosine.

Table 4**Stably Transfected COS7 Cells with PS Oligonucleotides**

Formulation	Lipid:Oligo Concentration	% Inhibition
DOTAP:DOPE:Amch (1:1:1)	3.3:1	25%
DODAP:DOPE:Amch (1:1:1)	3.3:1	20%
DOPE:Amch (1:1)	5:1	0%
LipofectAmine	Not Reported	59%
DOPE:Amch (1:1)	5:1	22%
DOPE:Amch (1:1)	1.25:1	16%
DOPE:Amch (1:1)	0.5:1	1%
Lipofectin	Not Reported	36%
LipofectAmine	Not Reported	60%
DOTAP:DOPE:Amch (1:1:0.5)	5:1	76%
DOPE:Amch (1:1)	5:1	16%
LipofectAmine	8:1	66%
DOPE:Amch (1:1.5)	5:1	35%
DOTAP:DOPE:Amch (1.3:1:1.2)	5:1	74%
DOTAP:DOPE:Amch (1:1:1.5)	5:1	73%

Table 5**Stably Transfected A10 Cells with PS Oligonucleotides**

Formulation	Lipid:Oligo Concentration	% Inhibition
DOPE:Amch (1:1.5)	5:1	0%
LipofectAmine	6:1	81%
DOTAP:DOPE:Amch (1.3:1:1.2)	5:1	16%
DOTAP:DOPE:Amch (1:1:1.5)	5:1	60%
LipofectAmine	6:1	48%
DOPE:Amch (1:1.5)	5:1	4%

10 ★ Significant inhibition was observed in most formulas.

5

EXAMPLE 4

A confocal microscopy study was undertaken to further verify cytoplasmic delivery of oligonucleotides using lipid constructs comprised of Aminomannose derivatized cholesterol, DOPE:Amch (1:1 molar ratio) and DSPC:chol (2:1 molar ratio) were prepared as in Examples 1 & 2. The

10 DOPE:Amch lipid constructs were incubated for 10 minutes at 65°C with a fluorescently labeled chimeric oligonucleotide having the sequence 5'-GGTATATCCAGTGATCTTCTTCTC-3' (SEQ ID NO.: 13). The lipid to oligonucleotide ratio was 5:1 w/w. DSPC:chol liposomes were prepared by hydrating the lipids with buffer containing the chimeric oligonucleotides and

15 sonication. Six flasks containing 10⁶ P1798 (lymphocarcinoma cells) cells in 10 ml complete Iscoves (10% Fetal Calf Serum, 1% penicillin/streptomycin) were incubated at 37°C, 5% CO₂. The cells were then incubated with 50 µg of oligonucleotide, either as free oligo or lipid associated oligo. Flasks 1, 2 and 3 were incubated with free oligo or oligo complexed with one of the two lipid

20 formulations overnight (16 hour incubation). Flasks 4, 5 and 6 were incubated the next morning with free oligo or oligo complexed with one of the two lipid formulations (2.5 hour incubation). Cells were removed at 2.5 and 16 hours. The cells were washed to remove unassociated liposomes/oligonucleotide and examined by confocal microscopy. For each sample, a confocal

25 microscopy series and corresponding bright field image were recorded. That is, a midsection fluorescence (top), bright-field (middle) and 3-d reconstruction (bottom) are displayed in figures 2-4. Figures 2A and 2B show rapid and intense fluorescence diffusing from the edge of the cells into the cytoplams. As the figures indicate, Amch:DOPE lipid constructs exhibit

30 rapid and concentrated fluorescence into the cytoplasm as shown by intense fluorescence in the cytoplasm whereas the cells incubated with free oligonucleotide and oligonucleotide associated with DSPC:chol constructs

- 5 shows very little fluorescence at all time periods.

EXAMPLE 5

A transfection study was carried out to further verify the ability of aminomannose derivatized cholesterol lipid constructs to deliver oligonucleotides into the nucleus of a cell. As noted above, LFA is currently
 10 used to transfect a plasmid, that is, introduce the plasmid into the nucleus of a cell so that it expresses a protein for which it codes. The plasmid was pG1035 which has a modified chloramphenicol acetyl transferase (CAT) gene inserted into pRc/CMV (Invitrogen). pG1035 was produced as follows. A modified chloramphenicol acetyl transferase (CAT) gene producing wild-
 15 type CAT protein was amplified in a PCR reaction using the following primers:
 5' primer:

Hind III

5' atc caa gct tcg acg aga ttt tca gga 3' (SEQ ID NO.: 14)

3' primer:

20 Not I

5' tta tgc ggc cgc tta cgc ccc gcc ctg cca 3' (SEQ ID NO.: 8)

The amplified fragment was cut using Hind III and Not I restriction enzymes and ligated into Hind III/Not I cut pRc/CMB (invitrogen) vector. The amino terminal protein and 5' nucleotide sequence of the CAT gene in pG1035 are:

25 Wild-type CAT:

5' +1 3'
 Met Glu Lys Lys Ile Ser Gly Tyr Thr Thr
 (SEQ ID NO.: 9)

uuu uca gga gcu aag gaa gcu aaa aug gag aaa aaa auc acu gga uau acc acc
 30 (SEQ ID NO.: 10)

It is known that LFA is optimized at a lipid:plasma w/w ratio of 6:1. The following lipid:plasmid constructs were formed: DOPE:Amch (1:1 molar ratio)

- 5 with a lipid plasmid ratio w/w of 10:1; DMPE:Amch (1:1 and 1:1.5 molar ratios)
with lipid plasmid ratio w/w of 10:1; DODAP:DOPE:Amch (1:1:1) at lipid
plasmid ratios w/w of 15:1 and 10:1. The DOPE:Amch lipid constructs were
1.14 times more efficacious than LFA. The DMPE:Amch constructs, 1:1 and
the 1:1.5, were 0.44 and 0.71 as effective as LFA respectively. The
10 DODAP:DOPE:Amch 15:1 and 10:1 formulas, were 0.02 and 0.01 as effective as
the LFA respectively. In all cases, the Aminomannose constructs were
effective in transfecting A10 smooth muscle cells.

EXAMPLE 6

- Lipid constructs were formulated as in Example 2 using the following
15 Oligonucleotides: cdc gene (rat) GTCCTCCATAGTTACTCA (SEQ ID NO.: 1);
PCNA gene (rat) GATCAGGCGTGCCTCAAA (SEQ ID NO.: 2); rev-AS (2328)
ACTCATTGATACCTCCTG (SEQ ID NO.: 3); rev-AS (2342)
AAACTCCGTGCGGACTAG (SEQ ID NO.: 4). Ten unheparinized rats were
treated with 10 μ M total oligo (antisense or reverse antisense) complexed with
20 DOPE:aminomannose (1:1) at a 5:1, 1.25:1 or 0.5:1 lipid:oligo w/w ratios and
sacrificed 2 weeks post-angioplasty. Eight heparinized rats were treated with
10 μ M total oligo (antisense or reverse antisense) complexed with
DOPE:aminomannose liposomes (1:1) at 5:1 lipid:oligo w/w ratio and sacrificed
2 weeks post-angioplasty.
- 25 A No. 2 French Fogarty catheter was used to induce vascular injury in
male Sprague-Dawley rats. The rats were anesthetized, and a cannula
introduced into the left common carotid via the external carotid artery. After
vascular injury of the common carotid, the distal injured segment was
transiently isolated by temporary ligatures. The lipid complex formulation
30 (0.25 ml) containing the antisense oligos (5 μ M of each) was infused into the
segment and incubated for 15 - 30 minutes at room temperature. Control rats
received an identical formulation containing reverse antisense oligos (rev-

- 5 AS). Following incubation, the infusion cannula was removed and blood flow to the common carotid restored by release of the ligatures. At 2 weeks after catheterization, rats were sacrificed and vessels perfused-fixed with 4% paraformaldehyde. Sections were analyzed for neointima formation, and data expressed as neointimal/medial area ratios and percent inhibition.
- 10 Unheparinized rats treated with DOPE:aminomannose lipid constructs formulated at 1.25:1 or 0.5:1 lipid:oligo w/w showed excellent inhibition of neointima formation as compared to rats treated with liposomes formulated with reverse antisense. Neointima formation was inhibited approximately 45% and 38% by the 0.5:1 and 1.25:1 constructs respectively. However, liposomes
- 15 formulated with 5:1 lipid:oligo w/w were not effective. 60-70% of the unheparinized rats showed moderate to severe thrombosis. Heparinized rats treated with the 5:1 constructs showed no thrombosis and no reduction in neointima formation when compared to heparinized rats treated with reverse antisense.

20

EXAMPLE 7

- Lipid constructs were prepared as in Example 2 and complexed with oligonucleotides as in Example 6. Rats were heparinized and treated with constructs complexed with cdc2 and PCNA (0.5:1 lipid:oligo w/w and 50 μ M oligo, 0.5:1 lipid:oligo w/w and 5 μ M oligo, 0.25:1 lipid:oligo w/w and 5 μ M
- 25 oligo) or reverse cdc2 and PCNA (0.5:1 lipid:oligo w/w and 5 μ M oligo) phosphorothioate oligonucleotides. The results of the study are listed below in Table 6. Unlike the previous study done with unheparinized rats (Example 6), only 1 rat in the entire study showed signs of thrombosis (rat treated with 0.25:1 lipid:oligo w/w and 5 μ M oligo). All rats treated with antisense
- 30 oligonucleotides showed a therapeutic response (treated arteries as compared to untreated control arteries).

5

TABLE 6

Neointima Formation in Treated vs. Untreated Control Arteries

	Treatment Group	Treated	Untreated	% Reduction	t-test
AS	0.5:1, 50 μ M	0.179 \pm 0.035	0.234 \pm 0.042	22.9%	0.013
AS	0.5:1, 5 μ M	0.153 \pm 0.044	0.250 \pm 0.053	38.7%	<10 ⁻⁴
AS	0.25:1, 5 μ M	0.158 \pm 0.028	0.257 \pm 0.034	38.5%	<10 ⁻⁴
rev AS	0.5:1, 5 μ M	0.181 \pm 0.022	0.183 \pm 0.018	None	0.834

EXAMPLE 8

Stock solutions of the oligonucleotides were prepared as followed:

- 10 antisense, c-myc:5'-AACGTTGAGGGGCAT-3' (SEQ ID NO.: 15) and nonsense c-myc:5'-CTGAAGTGGCATGAG-3' (SEQ ID NO.: 16) were dissolved in phosphate buffer to make a stock solution with a concentration of approximately 3.4 mg/ml. A lipid film was prepared containing hydrogenated soy phosphatidylcholine:chol:Amch in a mole ratio of 16:8:1 having a total
- 15 lipid concentration of 560 milligrams. One ml of the oligonucleotide stock solution was added to the lipid film and bath sonicated at a temperature of 40° C for 10 seconds. The resulting solution was put through a 4 cycle freeze-thaw procedure using liquid nitrogen. The resulting homogeneous solution was extruded first through a 0.8 μ M filter membrane (3 times) then extruded
- 20 through a 0.45 μ M filter (3 times) and finally extruded through a 0.2 μ M filter (3 times). The free oligonucleotides were removed by passing through a Sephadex G-50 column with a bed volume of about 18 ml. The vesicle volume was recovered in 5.5 ml of buffer. Control liposomes were prepared as above without oligonucleotides. The mean diameter of the control liposomes was
- 25 80.0 \pm 30 8.4nm. The mean diameter of the oligonucleotides containing liposomes were 210 \pm 80nm. The antisense c-myc and oligonucleotides

5 concentration was 50 µg/ml.

The liposomes containing oligonucleotides were tested against two strains of small cell lung carcinoma. The first strain was a cisplatin (cDDP), sensitive line (GLC4) and the second strain was a 5.6x cDDP resistant sub-line (GLC4-cDDP). In GLC4 and GLC4-cDDP there is a 40-60x *c-myc* oncogene
10 amplification with a corresponding *c-myc* mRNA and protein overexpression. The lipid concentration in the control group was 21.8 mg/ml and the lipid concentration in the liposome preparation containing the oligonucleotides was 10.3 mg/ml. Both cell lines showed growth inhibition after incubation with 10 and 20 µM antisense *c-myc* oligonucleotides in SCLC-medium, a
15 defined serum-free culture medium (heat-inactivated for 1 hour at 70° C). Thus, the remaining nucleases were heat-inactivated. In these cell cultures nonsense oligonucleotides did not have any inhibitory effect. The results are listed in Table 7. The results in Table 1 disclose that encapsulated oligonucleotides in liposomes allow for a 10 to 20 times reduction of antisense
20 *c-myc* oligonucleotides concentration needed to exert the same inhibitory effect in the GLC4 and GLC4-cDDP cell lines compared to the unencapsulated antisense *c-myc* oligonucleotides. Empty liposomes and nonsense oligonucleotides did not have an inhibitory effect.

5

TABLE 7

% Growth to control \pm sd (n=3)	GLC4	GLC4-cDDP
control	100	100
10 μ M as c-myc n-ODN	83.6 \pm 5.9	82.1 \pm 7.9
20 μ M as c-myc n-ODN	69.5 \pm 10.2	55.5 \pm 5.9
10/20 μ M ns c-myc n-ODN	99.7 \pm 6.1	100.3 \pm 6.1
1 μ M as c-myc in liposomes	70.3 \pm 9.0	62.1 \pm 8.6
2.5 μ M as c-myc in liposomes	56.9 \pm 10.3	31.3 \pm 15.5
empty liposomes	104.6 \pm 8.3	80.4 \pm 14.6

While the invention has been described with reference to specific
embodiments and examples, it will be appreciated that various changes and
10 modifications can be made without departing from the invention.

Although this specification has been disclosed and illustrated with
reference to particular applications, the principles involved are susceptible to
numerous other applications which will be apparent to those skilled in the art.

The invention is, therefore, to be limited only as indicated by the scope of the
15 appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: LIPID CONSTRUCTS FOR CYTOPLASMIC DELIVERY OF AGENTS

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/386,577
- (B) FILING DATE: 10-FEB-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GTCCTCCATA GTTACTCA

18

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

33

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GATCAGGCGT GCCTCAA

18

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACTCATTGAT ACCTCCTG

18

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AAACTCCGTG CGGACTAG

18

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TAGCTTCCTT AGCTCCTGCA T

21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TAGCTTCCGC AACTCTTGCA T

21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 72 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CACAAGCTTG TCGACTCGAG TGCAGGAGCT AAGGAAGCTA CCATGGAGAA GAAGATCACT 60

GGATATACCA CC

72

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TTATGCGGCC GCTTACGCCC CGCCCTGCCA

30

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Met	Glu	Lys	Lys	Ile	Ser	Gly	Tyr	Thr	Thr
1				5					10

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

UUUUCAGGAG CUAAGGAAGC UAAAAUGGAG AAAAAAUCA CUGGAUAUAC CACC

54

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

36

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Met	Glu	Lys	Lys	Ile	Ser	Gly	Tyr	Thr	Thr
1				5					10

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AGUGCAGGAG CUAAGGAAGC UACCAUGGAG AAGAAGAUCA CUGGAUAUAC CACC

54

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGTATATCCA GTGATCTTCT TCTC

24

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATCCAAGCTT CGACGAGATT TTCAGGA

27

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AACGTTGAGG GGCAT

15

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CTGAAGTGGC ATGAG

15

We claim:

1. A method for delivering a therapeutic or imaging agent into the cytoplasm of a cell comprising the administration to a mammal a lipid construct having an aminomannose-derivatized cholesterol moiety.
2. The method as recited in claim 1 wherein the aminomannose -derivatized moiety is 6-(cholest-5-en-3 β -yloxy)hexyl-6-amino-6-deoxy-1-thio- α -D-mannopyranoside.
3. The method as recited in claim 1 wherein the agent is an oligonucleotide.
4. The method as recited in claim 2 wherein the agent is an oligonucleotide.
5. The method of claim 3 wherein the oligonucleotide inhibits the growth of vascular smooth muscle cells.
6. The method of claim 4 wherein the oligonucleotide inhibits the growth of smooth muscle cells.
7. The method of claim 3 where the lipid construct comprises a cationic lipid wherein the total cationic lipid concentration is greater than 5% of total lipid.
8. The method of claim 4 where the lipid construct comprises a cationic lipid wherein the total cationic lipid concentration is greater than 5% of total lipid.
9. The method of claim 5 where the lipid construct comprises a cationic lipid wherein the total cationic lipid concentration is greater than 5% of total lipid.

10. The method of claim 6 where the lipid construct comprises a cationic lipid wherein the total cationic lipid concentration is greater than 5% of total lipid.
11. The method of claim 3 where the oligonucleotide inhibits the growth of cancer cells.
12. The method of claim 4 where the oligonucleotide inhibits the growth of cancer cells.
13. The method of claim 11 where the oligonucleotide has the sequence AACGTTGAGGGGCAT (SEQ ID NO.: 15).
14. The method of claim 12 where the oligonucleotide has the sequence AACGTTGAGGGGCAT (SEQ ID NO.: 15).
15. The method of claim 11 where the oligonucleotide has the sequence CTGAAGTGGCATGAG (SEQ ID NO.: 16).
16. The method of claim 12 where the oligonucleotide has the sequence CTGAAGTGGCATGAG (SEQ ID NO.: 16).
17. A lipid construct comprising an oligonucleotide which inhibits the growth of vascular smooth muscle cells wherein the construct comprises 6-(cholest-5-en-3 β -yloxy)hexyl-6-amino-6-deoxy-1-thio- α -D-mannopyranoside and DOPE:Amch in mole ratio of 1:1 wherein the lipid:oligonucleotide ratio is at or between about 0.5:1 and 1.25:1.
18. The construct as recited in claim 17 wherein said oligonucleotide is an

antisense oligonucleotide having a size up to 80 mer.

19. The construct as recited in claim 18 wherein said oligonucleotide has a size from 15 mer to 30 mer.

20. The construct as recited in claim 19 wherein said oligonucleotide comprises GTCCTCCATAGTTACTCA (SEA ID NO.: 1).

21. The construct as recited in claim 19 wherein said oligonucleotide comprises GATCAGGCGTGCCTCAAA (SEQ ID NO.: 2).

22. A method for inhibiting the growth of a cancer cell comprising the delivery of a lipid construct having an aminomannose derivatized cholesterol moiety wherein the construct contains an oligonucleotide which inhibits the growth of cancer cells.

23. The method as recited in claim 22 wherein the aminomannose-derivatized moiety is 6-(cholest-5-en β -ylozyl)hexyl-6-amino-6-deoxy-1-thio- α -D mannopyranoside.

24. The method of claim 22 wherein the oligonucleotide is antisense to bcl-1 mRNA.

25. The method of claim 23 wherein the oligonucleotide is antisense to the bcl-1 mRNA.

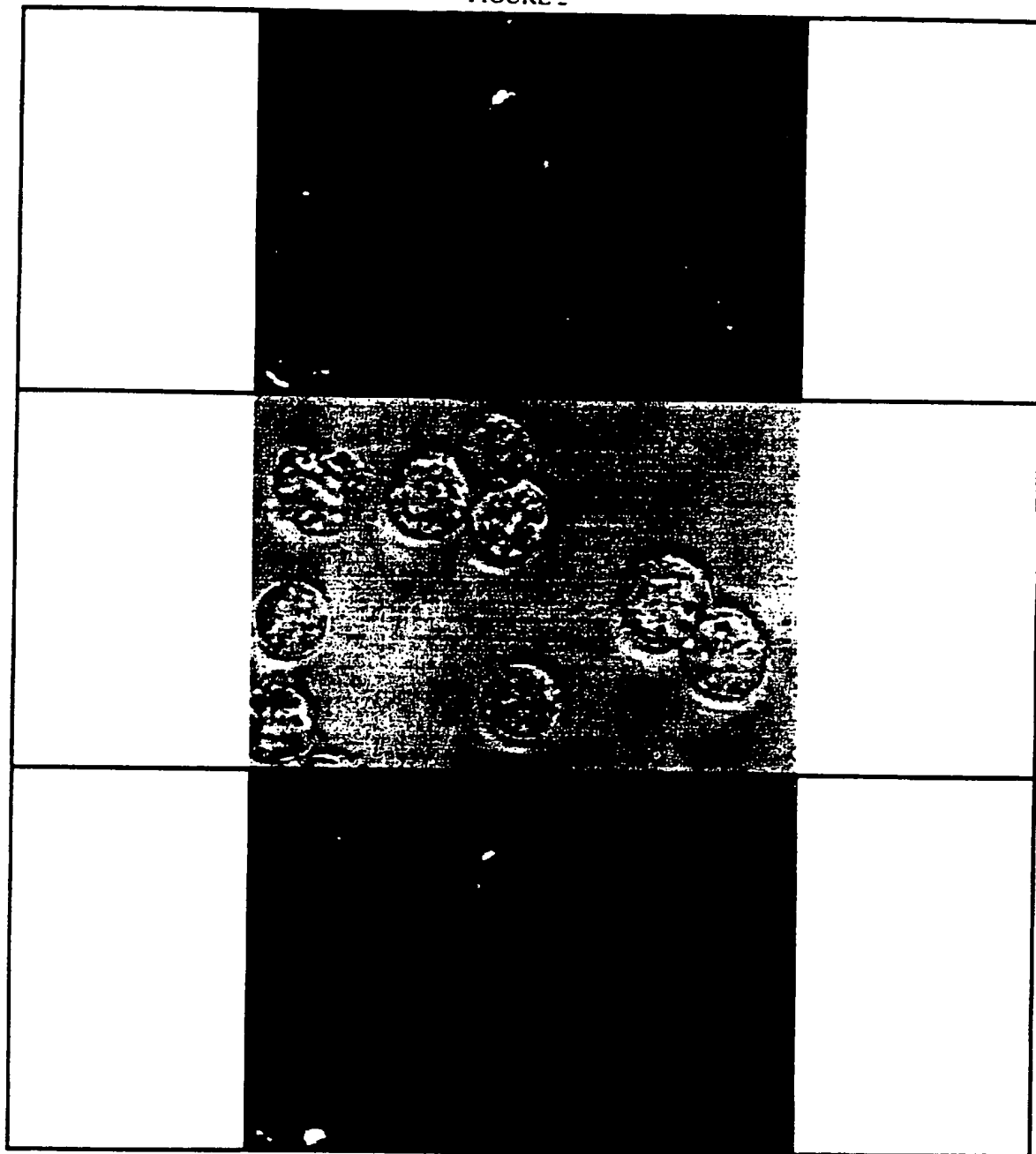
26. The method of claim 23 wherein the oligonucleotide is antisense to the c-myc mRNA.

27. The method of claim 22 where the oligonucleotide has the sequence AACGTTGAGGGGCAT (SEQ ID NO.: 15).
28. The method of claim 23 where the oligonucleotide has the sequence AACGTTGAGGGGCAT (SEQ ID NO.: 15).
29. The method of claim 3 wherein the oligonucleotide blocks cell-cycle regulatory genes.
30. The method of claim 4 wherein the oligonucleotide blocks cell-cycle regulatory genes.
31. The method of claim 29 wherein the cell-regulatory gene is a proliferating-cell nuclear antigen (PCNA).
32. The method of claim 30 wherein the cell-regulatory gene is a proliferating-cell nuclear antigen (PCNA).
33. The method of claim 29 wherein the cell-regulatory gene is p34cdc2 (cdc2).
34. The method of claim 30 wherein the cell-regulatory gene is p34cdc2(cdc2).
35. The method of claim 29 wherein the cell-regulatory gene is cdk2.
36. The method of claim 30 wherein the cell-regulatory gene is cdk2.

FIGURE 1



FIGURE 2

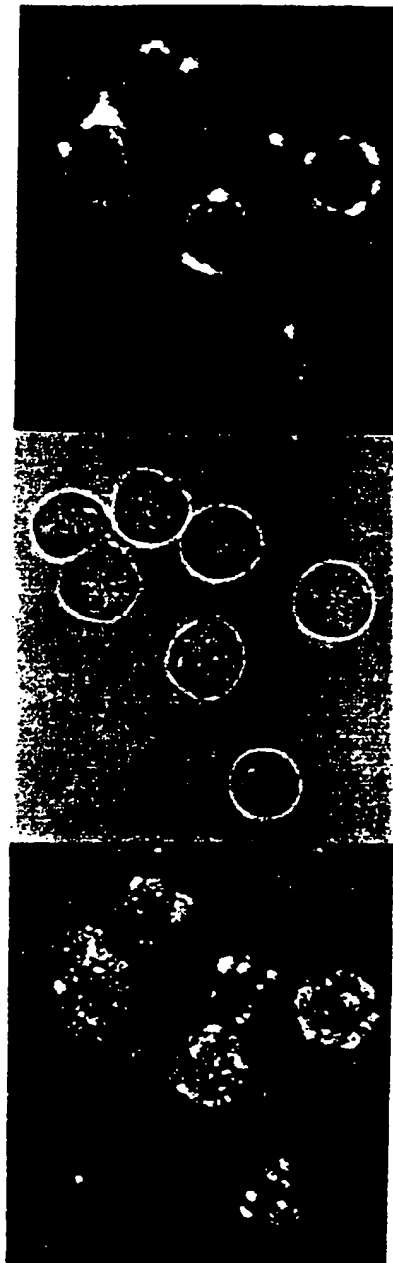


FREE OLIGO AT 2.5 HOURS

2/4

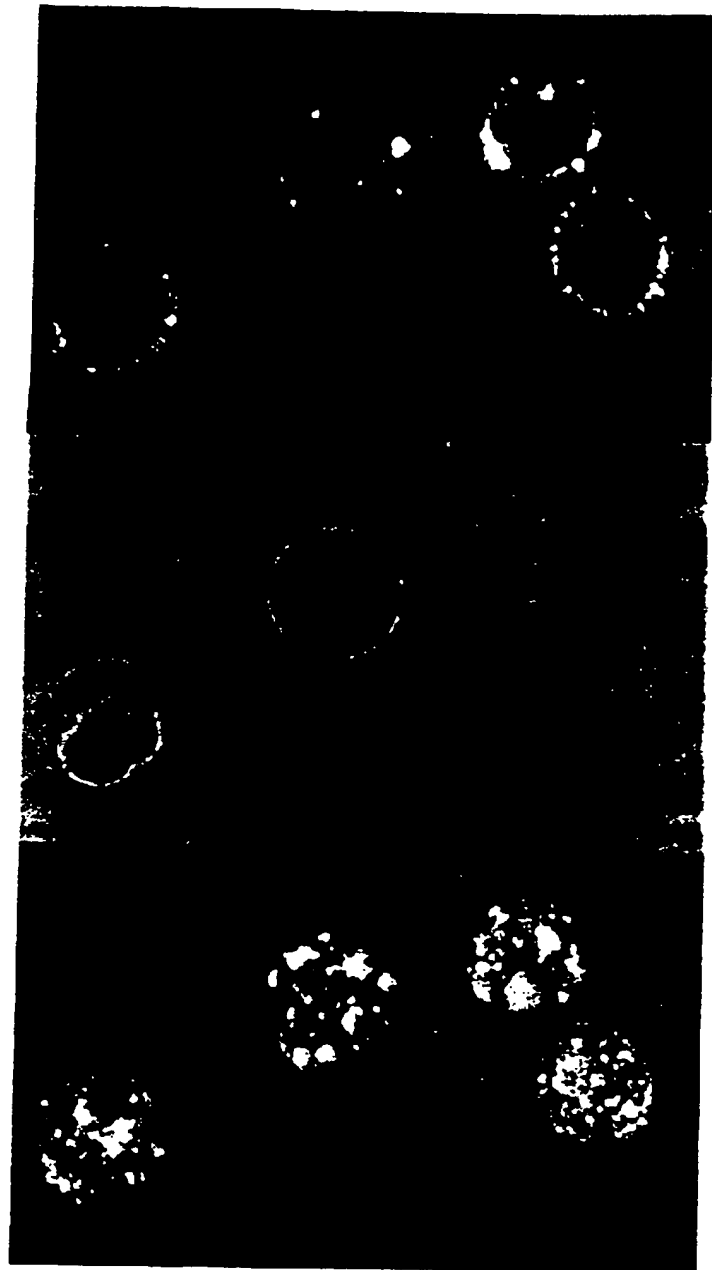
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FIGURE 3A



DOPE:Amch:Oligo
at 2.5 hours

FIGURE 3B



DOPE:Amch:Oligo
at 16 hours

FIG. 1A

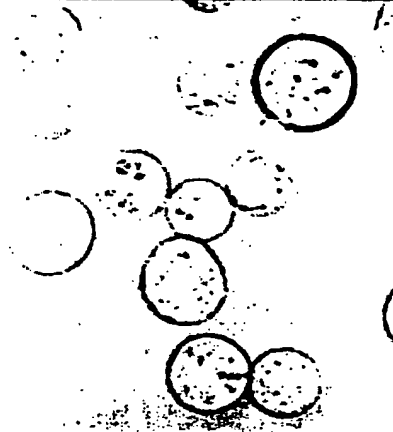


FIG. 1A: Electron micrograph of a cell surface showing viral particles (top) and a cell surface (bottom).

FIG. 1B

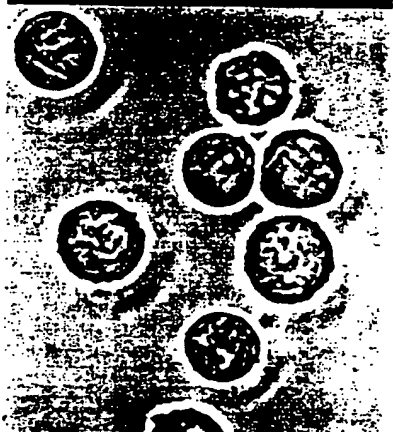


FIG. 1B: Electron micrograph of a cell surface showing viral particles (top) and a cell surface (bottom).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01960

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 9/127, 48/00

US CL : 424/450; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	US, A, 4,310,505 (BALDESCHWIELER ET AL) 12 January 1982, see entire document.	1-2 --- 3-36
Y	WO, A, 94/15646 (Thomas Jefferson University) 21 July 1994, see entire document.	11-16, 22-28
X	JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, issued February 1994, Male et al, "Receptor-assisted liposome uptake into vascular smooth muscle cells", page 188A, abstract no. 891-37.	1-2

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search

03 APRIL 1996

Date of mailing of the international search report

31 MAY 1996

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, Volume 21, No. 2, issued February 1993, Shi et al, "Maximizing Gene Transfer Into Vascular Smooth Muscle Cells", page 209A, abstract no. 900-37.	5-6, 9-10, 20-21, 29-36
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 198, No. 1, issued 14 January 1994, Abe et al, "Suppression of neointimal smooth muscle cell accumulation in vivo by antisense cdc2 and cdk2 oligonucleotides in rat carotid artery", pages 16-24, see entire document.	5-6, 9-10, 20-21, 29-36
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Volume 90, issued September 1993, Morishita et al, "Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia", pages 8474-8478, see entire document.	5-6, 9-10, 20-21, 29-36
Y	GASTROENTEROLOGY, Volume 106, issued April 1994, Nagano et al, "Growth inhibition of a gastric cancer cell line by antisense oligonucleotides to c-myc and bcl-2", page A419.	11-16, 22-28
Y	HUMAN GENE THERAPY, Volume 3, issued 1992, Stewart et al, "Gene Transfer In Vivo with DNA-Liposome Complexes: Safety and Acute Toxicity in Mice", pages 267-275, see entire document.	1-36

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